Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Infectious Canine Hepatitis Virus in Primary Canine Kidney Cell Culture

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Table of Contents

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live infectious canine hepatitis (ICH) or canine adenovirus (CAV) type 1 virus vaccines for viral content. Presence or absence of ICH is determined by cytopathic effect (CPE) in primary dog kidney (DKp) cell culture.

1.2 Keywords

Infectious canine hepatitis virus; ICH; canine adenovirus type 1; CAV; $TCID_{50}$; potency test; *in vitro* titration

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36° ± 2°C, high humidity, 5% ± 1% CO_2

2.1.2 Water bath,[2] 36° ± 2°C

2.1.3 Microscope,[3] inverted light

2.1.4 Vortex mixer[4]

2.1.5 Syringe,[5] 2 ml self-refilling, repetitive

2.1.6 Pipettor[6] with tips[7] and/or motorized microliter pipette[8]

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[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750 or equivalent
[2] Cat. No. 66648, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, Ny 11747-3157 or equivalent
[4] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[5] Wheaton®, Cat. No. 13-689-50C, Fisher Scientific Co., 319 W. Ontario, Chicago, IL 60610 or equivalent
[6] Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent
[7] Cat. No. RT-200, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent
[8] Cat. No. E2-250, Rainin Instrument Co. or equivalent

2.1.7 Micropipettor,[9] 300 µl x 12 channel

2.1.8 Pipette-aid[10]

2.2 Reagents/supplies

2.2.1 ICH Reference Virus,[11] Mirandola strain of ICH virus, Type 1

2.2.2 Monospecific antisera,[11] free of ICH antibody, that neutralize the non-ICH fractions present in multifraction vaccines, e.g. canine parainfluenza virus (CPI), canine parvovirus (CPV), canine distemper virus (CDV), etc.

2.2.3 Primary canine kidney cell culture,[12] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts[13]

2.2.4.2 2.2 g sodium bicarbonate ($NaHCO_3$)[14]

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamin[15] to 10 ml DW, heat to 60° ± 2°C until dissolved, then add to the solution in **Section 2.2.4.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DW, and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[16]

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[9] Finnpipettes®, Cat. No. NV204662D, Labsystems OY, Pulttitie 9, 00810 Helsinki 81, Finland or equivalent
[10] Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent
[11] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[12] ATCC CCL 81, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852
[13] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[14] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[15] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[16] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

2.2.4.6 Sterilize through a 0.22-µm filter.[17]

2.2.4.7 Aseptically add:

1. 25 units/ml penicillin[18]

2. 50 µg/ml gentamicin sulfate[19]

3. 100 µg/ml streptomycin[20]

2.2.4.8 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 940 ml MEM

2.2.5.2 Aseptically add:

1. 50 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine[21]

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)[22]

2.2.6.2 0.2 g potassium chloride (KCl)[23]

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[24]

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)[25]

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[17] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[18] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[19] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[20] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[21] 200 mM (100X) liquid, Cat. No. G-7513, Sigma Chemical Co. or equivalent
[22] Cat. No. 3624-01, J.T. Baker, Inc. or equivalent
[23] Cat. No. P217-500, Fisher Scientific Corp., 711 Forbes Ave., Pittsburgh, PA 15219-4785 or equivalent
[24] Cat. No. 3246-01, J.T. Baker, Inc. or equivalent
[25] Cat. No. M33-500, Fisher Scientific Corp. or equivalent

2.2.6.5 Dissolve reagents in **Section 2.2.6.1** through **Section 2.2.6.4** with 900 ml DW.

2.2.6.6 Add 1.03 g sodium phosphate, dibasic anhydrous (Na_2HPO_4)[26] to 10 ml DW, heat to 60° ± 2°C until dissolved. Add to the solution in **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$)[27] with 10 ml DW and add slowly to the solution in **Section 2.2.6.6** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW; adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.6.10 Store at 4° ± 2°C.

2.2.7 Cell culture plates,[28] 96 well

2.2.8 Polystyrene tubes,[29] 12 x 75 mm

2.2.9 Pipettes,[30] 10 ml

2.2.10 Reagent reservoir[31]

2.2.11 Syringe,[32] 1 ml tuberculin

2.2.12 Needles,[33] 18 ga x 1½ in

[26] Cat. No. 3828-01, J.T. Baker, Inc. or equivalent
[27] Cat. No. 4225-05, J.T. Baker, Inc. or equivalent
[28] Cat. No. 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[29] Falcon® 2058, Becton Dickinson & Co., 2 Oak Park, Bedford, MA 01730 or equivalent
[30] Falcon® 7530, Becton Dickinson & Co. or equivalent
[31] Cat. No. 4870, Costar Corp. or equivalent
[32] Cat. No. 309602, Becton Dickinson & Co. or equivalent
[33] Cat. No. 305196, Becton Dickinson & Co. or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by CPE.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of DKp cell culture plates (Test Plates)

Cells are prepared from healthy, confluent DKp cell cultures at second or third passage. On the day of test initiation, using the 12-channel micropipettor, add 200 µl/well of approximately $10^{4.7}$ to $10^{5.2}$ cells/ml suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 Test Plate for the controls and 1 Test Serial. Each additional Test Plate allows testing of 3 additional Test Serials. Incubate at 36° ± 2°C in a CO_2 incubator and use within 4 hr.

3.3.2 Preparation of ICH Reference Virus Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of ICH Reference Virus in a water bath.

3.3.2.2 Using the 2-ml self-refilling repetitive syringe, dispense 1.8 ml MEM into enough 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet, and label (e.g., 8 tubes labeled 10^{-1} through 10^{-8}, respectively).

3.3.2.3 With a 200-µl pipettor, transfer 200 µl of the ICH Reference Virus to the first tube labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 µl from the 10^{-1}-labeled tube **(Section 3.3.2.3)** to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Prepare dilutions of each neutralizing non-ICH antiserum according to the CVB-L Reference and Reagent sheet or manufacturer's instructions.

3.4 Preparation of the sample

3.4.1 Conduct the initial test of a Test Serial with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-ml syringe and an 18-ga x 1½-in needle, rehydrate a vial of the Test Serial with the provided diluent by transferring 1.0 ml for a 1.0-ml-dose vaccine, 0.5 ml for a ½-ml-dose vaccine, etc., into the vial containing the lyophilized Test Serial; mix by vortexing. Incubate for 15 ± 5 min at room temperature (RT) (23° ± 2°C).

3.4.2 For multifraction ICH vaccines, neutralize the non-ICH fractions with antiserum specific to each virus fraction.

3.4.2.1 Dispense 200 µl of each of the required neutralizing antiserum into a 12 x 75-mm polystyrene tube labeled 10^{-1} and q.s. with MEM to 1.8 ml. For example, to neutralize the 3 viral non-ICH components of a CDV/ICH/CPI/CPV vaccine, dispense 200 µl of each of the diluted CDV, CPI, and CPV antisera into the tube labeled 10^{-1}; add 1.2 ml of MEM to obtain a final volume of 1.8 ml.

3.4.2.2 Pipette 200 µl of the reconstituted Test Serial to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.

3.4.2.3 Incubate at RT for 30 ± 5 min.

3.4.3 For vaccines containing only the ICH fraction, the 10^{-1} dilution is prepared by adding 200 µl of the Test Serial to 1.8 ml of MEM in a 12 x 75-mm polystyrene tube, labeled 10^{-1}; mix by vortexing.

3.4.4 Serial tenfold dilutions

3.4.4.1 Using a 2-ml self-refilling, repetitive syringe, dispense 1.8 ml MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet; appropriately label (e.g., 8 tubes, labeled 10^{-1} through 10^{-8}, respectively).

3.4.4.2 Using a new pipette tip, transfer 200 µl from the tube labeled 10^{-1} to the next dilution tube labeled 10^{-2}; mix by vortexing.

3.4.4.3 Repeat **Section 3.4.4.2** to the remaining tubes, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the test

4.1 On the day of test initiation, label the Test Plates and inoculate each of 8 wells/dilution with 25 µl of the Test Serial, starting with the highest dilution (most dilute). In a similar manner, inoculate 8 wells of the ICH Reference Virus Control (dilutions 10^{-8} through 10^{-5} for the example in **Section 3.3.2.2**). Change tips between each unique sample (i.e., each Test Serial and the ICH Reference Virus Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-8} through 10^{-5}).

4.2 Eight uninoculated wells serve as negative cell controls.

4.3 Incubate the inoculated Test Plates in the CO_2 incubator for 11 days ± 1 day.

4.4 After incubation, read at 100X or 200X magnification on an inverted light microscope and examine cells for CPE characterized by cell rounding and lysis.

4.4.1 Wells displaying one or more CPE foci are considered to be positive for ICH.

4.4.2 Record results as the number of CPE-positive wells versus total number of wells examined for each dilution of the Test Serial and the ICH Reference Virus Control.

4.5 Calculate the ICH endpoints of the Test Serial and the ICH Reference Virus Control using the Spearman-Kärber method as commonly modified. The titers are expressed as $\log_{10}$, 50% tissue culture infective doses ($TCID_{50}$).

Example:

10^{-2} dilution of Test Serial = 8/8 wells CPE positive
10^{-3} dilution of Test Serial = 5/8 wells CPE positive
10^{-4} dilution of Test Serial = 1/8 wells CPE positive
10^{-5} dilution of Test Serial = 0/8 wells CPE positive

Test dose titer = (X - d/2 + [d * S]) where:

X = $\log_{10}$ of lowest dilution (2)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportion of CPE positive

$$\frac{(8+5+1)}{8} = \frac{14}{8} = 1.75$$

Test dose titer = (2 - 1/2) + (1 * 1.75)= 3.25

Adjust the titer to the Test Serial dose size by adding the $\log_{10}$ of the reciprocal of the Inoculation Dose divided by the Test Serial Dose where:

Inoculation Dose = amount of diluted Test Serial added to each well of the Test Plate

Test Serial Dose = manufacturer's recommended vaccination dose

Example:

ICH endpoint = 3.25 log

$\frac{0.025 \text{ ml inoculum}}{1\text{-ml dose}} = \frac{1}{40}$ = 1.6 log

Total = 4.85 log

Titer of the Test Serial is $10^{4.85}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 Valid assay

5.1.1 The calculated titer of the ICH Reference Virus Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution of the ICH Reference Virus Control must have 100% positive CPE (8/8), and the highest (most dilute) must exhibit no CPE (0/8).

5.1.3 The uninoculated cell controls must not exhibit any CPE, degradation, or cloudy media that would indicate contamination.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.3 In a valid test if the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Serial is considered **SATISFACTORY**.

5.4 In a valid test if the titer of the Test Serial is lower than the titer contained in the APHIS filed Outline of Production for the product under test, the Test Serial may be retested in accordance with 9 CFR, Part 113.8.b.

6. Report of test results

Results are reported as $TCID_{50}$ per dose of Test Serial.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.305, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral, GE (Ed.), *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg. 731.

7.3 Finney, DJ, *Statistical method in biological assay.* Griffin, London, 3rd ed., 1978, pg. 508.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. Minor technical changes from the superseded protocol have been made in the neutralization procedure of non-ICH viral fractions and the age range of cell culture at the time of inoculation.